

Errors in Counting Platelets in Hemodialysis Patients by Use of Optical Microscopy

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Abstract

The classical method for counting platelets (Thrombocytes) is use of two types of microscopy: Burcker Turk chambers (hemacytometer) and slides with peripheral blood smears. In the last ten years, a new method, flow cytometry with application of the impedance principle has been developed for CBC and counting of platelets.

Scope:

The current studies sought to compare the results of platelet counts from the optic microscope with the values obtained by use of flow cytometry and the impedance principle in samples obtained from patients before and after undergoing hemodialysis.

Methods:

Three methods were used to assess platelet counts of hemodialysis patients: optical microscopy, peripheral blood smear and use of the cytometry principle with impedance principle (VIC) by Coulter HNX hematological analysis.

The study was performed on 90 patients, 55 men and 35 women, ages 35-65 years (mean age 50), hospitalized over a 3 month period. The patients were analyzed once monthly, all at the same day, before and after dialysis.

The samples were assessed for platelet count by statistical parameters: $[SD = (X_i - X_m)^2 / n - 1]$; Accuracy: $(\%Diff = X_{average} - X_{target} / X_{mean} \times 100)$, with normal value until ± 25 and Z score $(Z = (X_{average} - X_{target}) / SD)$, with normal value until ± 2 , $R > 0.95\%$, for average platelets $150-400 \times 10^3/\mu l$, 95% CI.].

Results:

The comparison between the platelet counts on the Coulter HMX (mean value $\bar{X} = 233 \times 10^3/\mu l$; $p=0.028$; $SD=2$; $\%Diff=0.90$; Z score = -0.30) and by optical microscopy ($\bar{X} = 250 \times 10^3/\mu l$; $p=0.029$; $SD= 2.6$; $\%Diff = -3.6$; Z score =0.40) yielded similar values in a control group (120 male and female healthy subjects, ages 25-55 years (mean age 40). A similarity between results for the methods was also found for the platelet counts of hemodialysis patients assessed

using the Coulter HMX (Pre-dialysis, mean value $\bar{X} = 230 \times 10^3/\mu l$; $p=0.024$; $SD=3.45$; $\%Diff = -4.53$; Z score =2.5) and post-dialysis, (mean value $\bar{X} = 245 \times 10^3/\mu l$; $p=0.034$; $SD=2.1$; $\%Diff = 6.34$; Z score = 0.10). However, differences were observed by use of optical microscopy in pre-dialysis, (mean value $\bar{X} = 261 \times 10^3/\mu l$; $p = 0.020$; $SD=7.1$; $\%Diff= 5.90$; Z score=3.90) and post-dialysis, (mean value $\bar{X} = 167 \times 10^3/\mu l$; $p = 0.6$; $SD=4.2$; $\%Diff= -7.10$; Z score= -2.90)..

The performance of the devices was assessed by Z score = < 1 = optic performance; $1 < Z < 2$ = good performance; $2 < Z < 3$ = satisfactory performance and $Z > 3$ = unsatisfactory performance.

The platelet count determined on the peripheral blood smear was used to complement data from the quantitative methods and provided morphological information.

Conclusion:

The methods used to assess platelet counts of hemodialysis patients, optical microscopy, peripheral blood smear and use of the cytometry principle with impedance principle (VIC) have had appropriated results with samples from normal subjects but the accuracy of the automatic method ensures a high quality count of hemodialysis patients.

Keywords

Variation Coefficient; $\%Diff$ -differential Percent; SD-standard Deviation; VCS Technology -Volume; Conductivity and Scatter Light; WOC Channel-white Cell Optical Count

Introduction

In order to avoid systematic errors during platelets(Thrombocytes) count by optical microscopy, a method of direct counting in the Burker-Turk chamber(hemacytometer) has been recommended for use in parallel with determination of the number of Thrombocytes counted on peripheral blood smear, (by optical microscopy). Calculation of the platelets counted in the Burker-Turk chamber considers the height of the chamber and the surface of the middle

square of the chamber to yield a value of 0.2mm^2 (Brecher G, Cronkite EP, 1995).

The calculation formula for hemacytometer cell counts determines the number of cells within $1\mu\text{L}$ (1mm^3) of blood. To make this determination, the total number of cells counted must be corrected for the initial dilution of blood and the volume of diluted blood used. The standard dilution of blood for platelet counts is 1:100; therefore the dilution factor is 100. The volume of diluted blood is based on the area and depth of the counting area. The area counted is 2mm^2 and the depth is 0.1mm ; therefore the volume factor is 0.2mm^3 . Total number of cells counted $\times$ dilution factor $\times$ $1/\text{volume factor} = \text{cells}/\text{mm}^3$. ($\text{Cells}/\text{mm}^3 = \text{cells}/\mu\text{L}$ or $\text{cells}/\mu\text{L} \times 10^3 \mu\text{L}/\text{L} = \text{cells} \times 10^9/\text{L}$).

A simple way to calculate platelet counts on the hemacytometer is to multiply the average platelet count between the two sides of the chamber by 1,000. For example, in a case: $68 \text{ platelets counted}/2\text{mm}^2 \times 1,000 = 68,000 \text{ platelets}$.

Direct microscopy of the blood smear yields the number of thrombocytes count by counting those found between 1000 erythrocytes (5 microscopic fields of 200 red cells) multiplied by the number of erythrocytes/ mm^3 and then divided /1000) with the results expressed as platelets/ mm^3 . The estimate of platelet count from slides uses a semi-quantitative method, whereby 1 platelet / oil immersion field is equivalent to $20,000 \text{ plt}/\text{mm}^3$, [Figure 1].

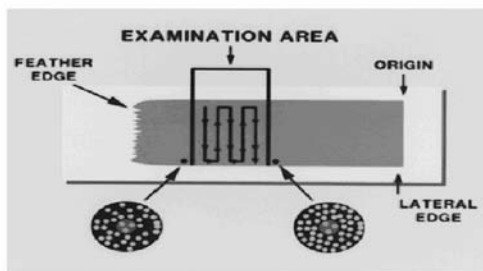


FIGURE 1 AREA EXAMINATION FOR PLATELETS COUNT ON BLOOD SMEAR

Counting thrombocytes on slide from peripheral blood smear is necessary in quantitative platelet disorders, as isolated thrombocytopenia: immune versus non-immune, thrombocytopenia associated with other hematological abnormalities or in differential diagnosis with platelet clump, thrombocytosis and qualitative disorders, as giant platelets (megathrombocytes), platelet inclusion or granule abnormalities, bizarre in shape and size.

In optical microscopy, one assesses a panoptic colored blood smear under the immersion objective ($100\times$). Most platelets have a dendritic aspect and fringe-like extensions. Normal platelets have diameter of 2-4 microns on the blood smear with 70% alone, 20% in groups of 2 or 3 and 10% in larger groups or "big pools". Correctly executed blood smear reveal microscopic fields on the oil-immersion objective with an average of 10 platelets as either isolated or grouped. Visualization of <5 platelets on the microscopic field connotes thrombocytopenia while >40 indicates thrombocythemia (Moreno A, Menke D, 2002).

Platelets are typically disk-shaped with a more dense central (granular) area and a peripheral (crystalline) area with functional dendritic fringes (Bennett JM, Rogers G, 2006). If activated by toxic metabolic factors, platelets become more spherical, which can yield a decrease in the intensity of the image in the microscopic lenses, due to light transmission and diffusion through samples. When platelets are activated, they become spherical with a hypogranular cytoplasm and release small particles. This may lead to the erroneous detection of platelets when using the microscopy owing to their deformed morphology. Recognizing erroneous results of platelet counts is especially critical for a consistent decision in the diagnosis of disseminated intravascular coagulation (DIC) and for clinical decision making regarding transfusion. The platelet count is an indispensable parameter in the DIC scoring system proposed by the International Society on Thrombosis and Haemostasis Sub-Committee of the Scientific and Standardization Committee on DIC, in which platelet counts of less than $100 \times 10^3/\mu\text{L}$ ($100 \times 10^9/\text{L}$) and less than $50 \times 10^3/\mu\text{L}$ ($50 \times 10^9/\text{L}$) would score 1 and 2 points, respectively (Kim YS et al. 2010, Segal HC, et al 2005).

It is interesting that platelet activation markers were associated with the severity of DIC and erroneous platelet counts, suggesting that platelet activation is a potential source for the inter-method variation in platelet counts. More attention needs to be given to improve the accuracy of platelet counts, especially in clinical conditions with high levels of platelet activation.

In chronic renal failure (CRF), the peripheral blood smear can reveal activated thrombocytes with fingers (burr cells) as isolated cells or organized in groups. By contrast, with diabetic ketoacidosis, one can see the reverse phenomenon, thrombocytes that are isolated, with round shape form and without activated fringes.

Figure 2. Qualitative platelets disorders: platelets with abnormality bizarre in shape and size.

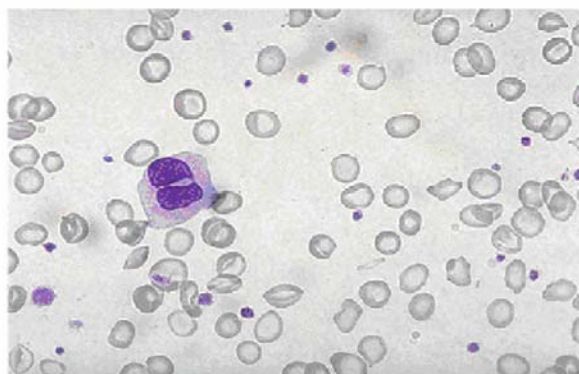
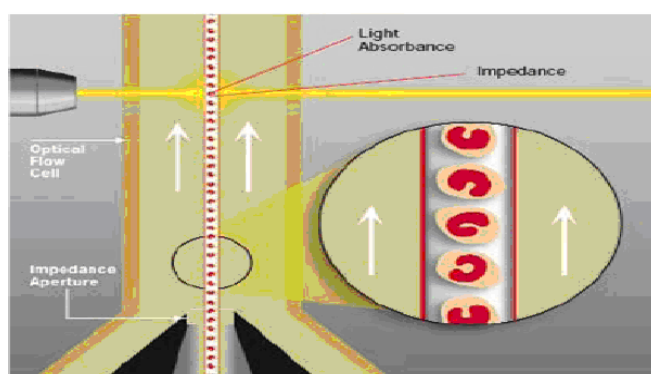


Figure 2 Qualitative platelets disorders: platelets with abnormality bizarre in shape and size

It is well known that white light is comprised of luminous waves with different wave lengths of 750-250 nm. Optical microscopy uses light diffraction but can have light reflection, refraction, diffusion and dispersion phenomena, especially through media with non-homogenous densities. Thus, it has been recommended that one conducts platelet counts using phase-contrast microscopy, which helps eliminate such light interference phenomena because the image is formed by a diffraction process in two stages: incident light diffraction and diffraction of the light refracted in the objective.

Figure 3. WOC technology: the laser light measures cellular elements in 4 specific angles. The zero angle measures the dimension of cells and impedance is used to count RBC and platelets corpuscles.



Graphic 5 WOC channel (White Cell Optical Count) is used for counting blood cells and differential count by laser technology. The result of this volumetric compensation is a pair of measurements that are very powerful, and unique to Beckman Coulter.

The optical conventional techniques used for platelet counting have limits that are influenced by the human eye, especially for detection of objects <5 microns.

Thus, the modern trend is to replace optical systems and introduce some electronic optical systems. Electronic microscopy with Beta rays and wave lengths thousands of times smaller than the white light gives a higher power of resolution and thus, analyzers well-suited for platelet count in biological fluids are ones that use either of two methods: WCS technology of impedance (Hennessy M, et al. 2003, Hickerson DH et al. 2002, Hervig T, et al. 2004, Harrison P, 2000).

In WCS, the fat within the cell membrane behaves as an object that facilitates generation of an electronic impulse with the amplitude proportionate as the cell volume and helps create a potential difference next to the count cleft. Since VCS technology includes a highly accurate measure of cell volume, we can use this information to correct the conductivity and scatter signals. The result of this volumetric compensation is a pair of measurements that are very powerful, and unique to Beckman Coulter. The HMX Coulter Analyzer utilizes the Coulter principle to provide cellular information for the complete WBC differential. The system measures the amount of light "lost" due to diffraction and absorbance as compared to full transmission when no cell is present. The collected signals are converted into voltage pulses and are processed. The size and shape of the voltage pulses are equivalent to the unique nuclear and morphologic structure of the cells being analyzed conductivity offers information about opacity, which is directly proportional to cell density. In WOC technology the laser light measures cellular elements in 4 specific angles and every angle of light scatter from 0° through 90° is influenced by cellular size. The low angles are the most affected, and are often used as an indirect estimation of cellular size. The zero angle measures the dimension of cells and impedance is used to count RBC (corpuscles > 36 fL) and platelets (corpuscles between 2-20 fL).

Scope

These studies were compared with compared the results of platelets counting by optic microscopy and from hematological analyses with flow cytometry and the impedance principle, in samples obtained from patients undergoing hemodialysis, before and after dialysis.

Methods

The prospective study of laboratory was performed on 90 known patients with chronic kidney diseases (CKD)

complicated with chronic renal failure(CRF), admitted in hospital, prior to undergoing schedules of dialysis, 55 men and 35 women, in average ages 35-65 years (mean, age 50, SD= +_2). The patients were analyzed once a month, all at the same day, to connection and after connection of hemo-dialysis schedules, in medical internal department. All the patients who investigated for complete blood cells count on a hematological analyzer Coulter HMX and for CFR diagnosis, usually and specific biochemical tests: Glucose, Urea nitrogen, Creatinine, Sodium, Potassium, E CO₂, on a dry chemistry analyzer Vitros 700(Ortho Diagnostics), Johnson & Johnson. [Table].

Platelet count was determined using three methods: optical microscopy, peripheral blood smear and by employing the cytometry principle with Coulter technology (VCS) which were statistically analyzed as follows: $[SD = \frac{1}{n-1} \sum (X_i - \bar{X})^2]$; Accuracy (%Diff = $(\bar{X} - \text{target}) / \bar{X} \times 100$, normal value until +_25) and Z score($Z = (\bar{X} - \text{target}) / SD$, with normal value until +_2), $R > 0.95\%$ for average platelets $150-400 \times 10^3/\mu\text{L}$, 95% CI.].

Results:

The comparison between the platelet counts on the Coulter HMX (mean value $\bar{X} = 233 \times 10^3/\mu\text{L}$; $p=0.028$; $SD=2$; % Diff=0.90; Z score = - 0.30) and by optical microscopy ($\bar{X} = 250 \times 10^3/\mu\text{L}$; $p=0.029$; $SD= 2.6$; %Diff = -3.6; Z score =0.40) yielded similar values in a control group (120 male and female healthy subjects, ages 25-55 years(mean age 40). For the dialysis patients, we found that results for platelet counts with the Coulter HMX, before and after hemodialysis were similar: (pre-dialysis mean $\bar{X} = 230 \times 10^3 \mu\text{L}$; $p=0.024$; $SD=3.45$; % Diff = -4.53; Z score =2.5; post dialysis mean $\bar{X} = 245 \times 10^3/\mu\text{L}$; $p=0.034$; $SD=2.1$; %Diff = 6.34; Z score = 0.10) but differences appeared if counting was done using optical microscopy (pre-dialysis mean $\bar{X}=261 \times 10^3/\mu\text{L}$; $p = 0.020$; $SD=7.1$; %Diff= 5.90; Z score=3.90); post-dialysis mean $\bar{X} = 167 \times 10^3/\mu\text{L}$; $p = 0.6$; $SD=4.2$; %Diff= -7.10; Z score= -2.90). Table 2

The latter results may be attributable to the variability of plasma osmolality in the samples taken from the patients with chronic renal failure: Glucose (98mg%; 5.44mmol/L; $SD=2.80$); Urea nitrogen (112 mg%; 40 mmol/L; $SD = 2.40$); Creatinine (5.5 mg%; 4.85 mmol/L); $SD=0.15$); Sodium (170 mmol/L; $SD=0.14$); Potassium (14.5 mmol/l; $SD=2.88$); E CO₂ (11 mmol/L; $SD=0.26$).

The performance of devices used was assessed by Z score = < 1 = optic performance; $1 < Z < 2$ = good

performance; $2 < Z < 3$ = satisfactory performance and $Z > 3$ =unsatisfactory performance. In parallel, we assessed platelet count using the peripheral blood smear and found that it provided information that was complementary to the other methods, especially with respect to morphological aspects of platelets.

Discussion:

The main elements that maintain plasma osmolality in normal individuals (310 Osm/l) are Na, K, urea and glucose. Serum osmolality is normal whenever the osmotic pressure set by urea and glucose is negligible and the Na⁺ concentration can largely define osmolality [$\text{Osm} = 2.1 \times \text{conc Na mEq/L}$]. Whenever the level of plasma urea or glucose is high, the osmolality becomes: $2.1 (\text{Na} + \text{K}) \text{ mmol/L} + \text{urea mg\%} / 2.8 + \text{glucose mg\%} / 18.02$, result expressed in Osm/L (Mehdi R, Kiarash RK, 2005).

In metabolic states with high osmolality (e.g., from chronic renal failure), errors in platelet counts occur in optical microscopy due to the double refraction phenomenon. This phenomenon occurs because particles $< 5\mu$ create reflection, refraction, diffusion and diffraction of light through environments with different properties (ϵ) and in solutions with higher osmolality. The diffraction of rays by objects $< 5\mu$ is not sufficiently dispersed and only a part of the issued light falls on the object from the objective of microscope. The angle comprised between the rays which delimit the light cone represents the numerical aperture(A) and the resolution power or the spectral separation power, dependent of light diffraction (D), light wave length (L) and numerical aperture (A) , [$D = L / A$], (Piston DW, 1998).

Optical instruments contain light separation media that are non-homogenous, including glass (ocular, objectives, prisms, air) and thus yielding

losses in the intensity of the incidence, reflection, refraction and diffraction rays through the media crossed by them. Considering the expression: $S = [n_1 - n_2/n_1 \times n_2]^2$, where "n represents the refraction index from the environment, the losses of the incidental ray, because of interference, is 4% from the intensity of incidental fascicle (Sterian P, 2008).

Platelets with dimensions $< 2\mu$ met by light rays, with a very high speed of propagation through liquid environments may not be seen in optical microscopy if increased osmolality concentrations are present. In accordance with Huygens interference principle, clefts S1 and S2 become secondary oscillation sources

(Foster P. 2010). The sources of secondary vibration of the light generated waves can overlap between the interference areas and fringes, thus yielding what is termed as the interference domain. Thus, the average of the intensity values of the object light image in the ocular may have the range between 0 and $4 \cdot e''$ ($e = 1/4nS$) in the minimal, respectively, maximum interference phase. The minimal intensity state of the light reflected on the object in order to create its reversed image in the ocular leads image loss for the human eye.

The normal thrombocytes having the diameter of 2-4 microns, create reflection, refraction, diffusion and diffraction of light through microscope become more or less suitable to manual counting. There has been some debate over which counting principle, between the impedance and optical methods, measures platelet counts more accurately. Some studies suggested that the accuracy of the optical methods was superior for thrombocytopenic specimens, while recent studies demonstrated the impedance method to be more accurate for samples from patients undergoing cytotoxic chemotherapy (Briggs C et al, 2007). More attention needs to be given to improve the accuracy of platelet counts, especially in clinical conditions with high levels of platelet activation.

Figure Captions

Figure 1. Area examination for platelets count on blood smear

Figure 2. Qualitative platelets disorders: platelets with abnormality bizarre in shape and size.

Figure 3. WOC technology: the laser light measures cellular elements in 4 specific angles. The zero angle measures the dimension of cells and impedance is used to count RBC and platelets corpuscles.

Table Captions

Table 1. Values of biochemical and hematological parameters in blood samples from patients with Chronic Renal Failure, undergoing the schedules of dialysis

Table 2 Assessment of performances for methods used in platelets count to patients with Chronic Renal Failure, undergoing dialysis

Conclusions

We analyzed blood obtained from normal subjects and patients undergoing hemodialysis in order to compare

3 methods for counting platelets: microscopy, peripheral blood smear and Coulter HMX automatic analyzer. We found that although the used methods have had appropriated results to the samples from normal subjects and that the accuracy of the automatic method ensures a high quality count but apparently not so, for patients post-dialysis. Examination of the peripheral blood smear appears to offer important advantages, in particular for dialysis patients, so as to assess for qualitative as well as quantitative changes in platelets in such patients.

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